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Genetic Evidence for a Long-Range Activity That Directs Pollen Tube Guidance in Arabidopsis

Martin Hülskamp,[a,b] Kay Schneitz,[a] and Robert E. Pruitt [a,1]

[a] Department of Molecular and Cellular Biology, The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138

[b] Institut für Entwicklungsgenetik, Eberhard-Karls Universität, Spemannstrasse 37-39, 72076 Tübingen, Germany

The fertilization process of plants is governed by different kinds of cell-cell interactions. In higher plants, these interactions are required both for recognition of the pollen grain by the female reproductive system and to direct the growth of the pollen tube inside the ovary. Despite many years of study, the signaling mechanisms that guide the pollen tube toward its target, the ovule, are largely unknown. Two distinct types of principles, mechanical and chemotropic, have been suggested to account for the directed growth of the pollen tube. The first of these two types of models implies that the guidance of the pollen tube depends on the architecture and chemical properties of the female reproductive tissues, whereas the latter suggests that the ovule provides a signal for the target-directed growth of the pollen tube. To examine such a role for the ovules, we analyzed the growth path of pollen tubes in mutants defective in ovule development in Arabidopsis. The results presented here provide unique in vivo evidence for an ovule-derived, long-range activity controlling pollen tube guidance. A morphological comparison of the ovule mutants used in this study indicates that within the ovule, the haploid embryo sac plays an important role in this long-range signaling process.

INTRODUCTION

Cell–cell interactions play a fundamental role in a variety of developmental processes in higher organisms. Such interactions may take place either between neighboring cells or between cells that are separated by some distance. A particular class of the latter type of interactions involves the guided growth of cells toward a target, as exemplified by nerve growth cone behavior (Bixby and Harris, 1991) and by pollen tube guidance during the fertilization process in higher plants (Heslop-Harrison, 1987; Lord and Sanders, 1992; Pruitt and Hülskamp, 1994). Whereas target-directed cell growth in neural systems is relatively well characterized, the mechanism that guides the growth of the pollen tube toward the ovule has yet to be defined.

The fertilization process in the plant Arabidopsis is an ideal model system in which to study cellular interactions that take place between cells in intimate contact and the guided growth of cells toward a target. The process begins when a pollen grain is captured by a papillar cell; one or more recognition events between the pollen and the papillar cell are required to trigger the hydration of the pollen grain (Heslop-Harrison, 1987; Nasrallah and Nasrallah, 1993; Pruitt and Hülskamp, 1994). If recognition interactions define the resident grain as being compatible, it will germinate to produce a pollen tube (Elleman et al., 1992; Pruitt and Hülskamp, 1994). Subsequent to the germination of the pollen tube, four different growth phases of the tube can be distinguished (Figure 1). (1) The emerging pollen tube penetrates the cuticle of the papillar cell through a "foot" of coating and enters the space between two cell wall layers (inner layer I and outer layer II) (Elleman et al., 1992). (2) At the base of the papillar cell, the pollen tube enters the transmitting tract, which is a specialized tissue within the septum that supports pollen tube growth in the ovary. Here, the pollen tube commences intercellular growth and continues to grow basipetally in a straight course. (3) Eventually, the pollen tube emerges from the transmitting tract onto the surface of the septum. (4) On the surface of the septum, the growth behavior changes dramatically in that the growing pollen tube now curves back and forth (Hill and Lord, 1987; Pruitt and Hülskamp, 1994). The pollen tube then proceeds to a funiculus and ultimately to the micropyle of an ovule. Here, the pollen tube delivers the two sperm cells to the embryo sac, and the actual event of double fertilization takes place.

The mechanism of pollen tube guidance in the four different growth phases is poorly understood. In principle, two mechanisms for guided growth of cells may be distinguished. The growth can be guided mechanically by the architecture of the tissues or chemotropically either by target-generated diffusible signals or by defined "tracks" of signals. Both types of mechanisms have been proposed to explain the high efficiency with which pollen tubes find the ovules (Mascarenhas and Machlis, 1962; Heslop-Harrison and Heslop-Harrison, 1986; Heslop-Harrison, 1987). The chemotropic model implies

[1] To whom correspondence should be addressed.

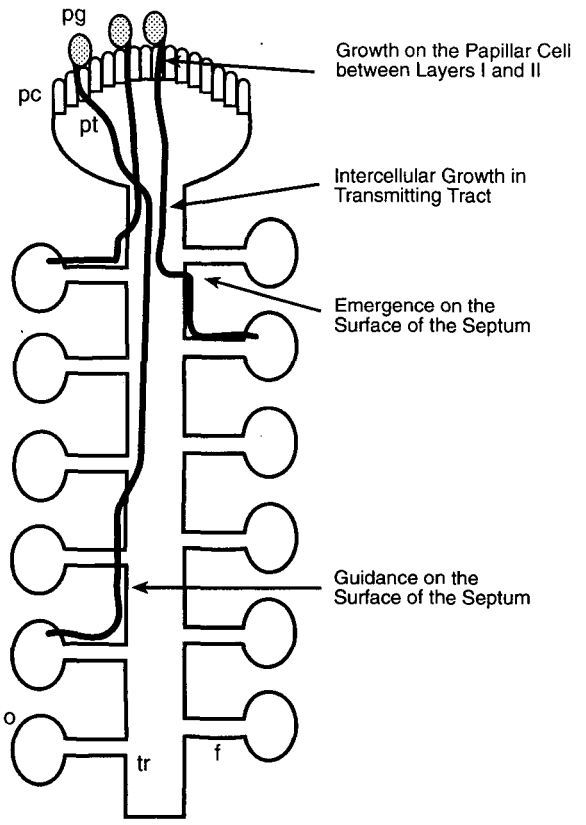

Figure 1. Schematic Representation of Pollen Tube Growth in the Pistil.

This schematic drawing depicts the four different growth phases of the pollen tube. pc, papillar cell; pg, pollen grain; pt, pollen tube; o, ovule; tr, transmitting tract; f, funiculus.

a role for the ovules in the control of pollen tube guidance. We therefore took a genetic approach to investigate the potential role of the ovules in directing the pollen tube by analyzing the pollen tube path in mutants defective in ovule development in Arabidopsis.

RESULTS

Pollen Tube Guidance in the Wild Type

To understand the mechanisms of pollen tube guidance, we addressed two questions concerning the growth path of the pollen tube in wild-type pistils. Where along the length of the transmitting tract do the pollen tubes emerge, and what influence, if any, does the number of participating pollen tubes have on the pattern of emergence? Is there a strict positional dependence between the emergence point of a given pollen tube and the ovule that is its ultimate target?

To analyze the growth path of pollen tubes during the different growth phases, we used a combination of light and electron microscopy. The growth path of pollen tubes inside the ovary can best be seen in whole-mount preparations of cleared, aniline blue–stained pistils (Martin, 1959), as shown in Figure 2A. We developed a modified whole-mount aniline blue staining protocol to distinguish selectively only those pollen tubes that have emerged onto the surface of the septum. This allowed us to follow the growth of individual pollen tubes from the point of their emergence to the target micropyle (Figure 2B). Three-dimensional aspects of pollen tube growth on the surface of the septum were analyzed by scanning electron microscopy (Figures 2C and 2D).

The emergence pattern of pollen tubes within the pistil was analyzed in noncleared, aniline blue–stained whole-mount preparations of ovaries carrying small numbers of pollen tubes (see Figure 2B). The site of pollen tube emergence was determined using the rows of ovules to define position within the ovary (Figure 3A). In ovaries bearing only one to three pollen tubes, most pollen tubes appear at the position of the first ovule, whereas more basally in the ovary, progressively fewer pollen tubes emerge on the surface. A similar pattern was obtained for ovaries with four to six or seven to 10 pollen tubes. However, the strong preference to emerge very close to the stigma is slightly reduced when more pollen tubes are present.

The selection of a particular ovule as a target by a pollen tube after emerging on the surface of the septum was also analyzed using the same method. Table 1 summarizes which ovules are targeted by the pollen tubes relative to their emergence points. Many pollen tubes (39 to 46%) grow directly toward the next available ovule. However, a substantial number of pollen tubes ignore one or more ovules before turning toward the ovule they target. As can be seen from Table 1, no significant differences in growth behavior were observed for ovaries containing different numbers of pollen tubes.

Phenotypic Description of Ovule Mutants

To determine whether and at which steps the pollen tube path may be influenced by the ovules, we investigated pollen tube behavior in four sporophytic recessive female-sterile mutants that differ in the extent to which either the sporophytic or the gametophytic tissue of the ovule is affected. In *bell* (*bel1*) and *short integuments* (*sin1*) mutants, both the sporophytic tissue and the embryo sac are affected (Robinson-Beers et al., 1992; Modrusan et al., 1994; Figures 4B and 4C). In *sin1* mutants, megasporogenesis appears to be blocked at meiosis; hence, no embryo sac is generated. In *bel1* mutants, some aberrant gametophyte development takes place (Robinson-Beers et al., 1992; Modrusan et al., 1994). In two recently isolated mutants, 47H4 and 54D12, the sporophytic tissue of the ovule and pistils is apparently normal (K. Schneitz, M. Hülskamp, S.D. Kopczak, and R.E. Pruitt, manuscript in preparation). With respect to embryo sac development, however, they differ significantly. In 47H4 mutants, no embryo sac develops (Figure 4D). In 54D12 mutants, the extent of ovule development varies in that either

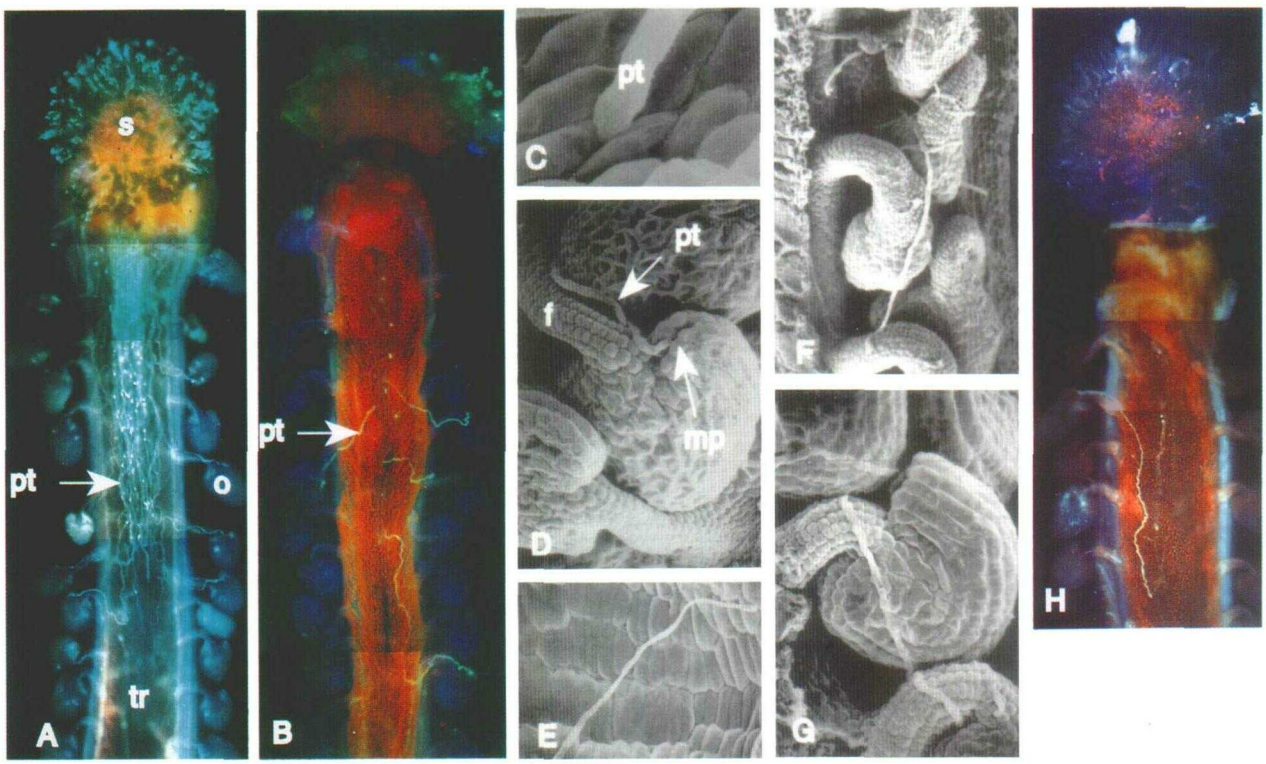

Figure 2. Pollen Tube Guidance in Ovaries of Wild-Type and Ovule Mutant Plants.

Composite fluorescence micrographs are shown in **(A)**, **(B)**, and **(H)**, and scanning electron micrographs are presented in **(C)** to **(G)**. For a description of the ovule mutants in **(E)** to **(H)**, see Figure 4.

(A) A whole Arabidopsis wild-type ovary that has been dissected free from the ovary wall. The stigma is oriented toward the top. The ovules are folded to the side of the transmitting tract. The pollen tube path can be seen by the blue-green fluorescence of the pollen tubes. The tissue was cleared prior to the staining to reveal the pollen tubes inside and outside the transmitting tissue.

(B) A whole-mount wild-type ovary that has not been cleared prior to staining. The staining of pollen tubes within the transmitting tract is quenched by the fluorescence of the chlorophyll. Thus, the path of the pollen tube can be detected only after its emergence on the surface of the septum. This ovary shows the path of four pollen tubes. Three grow from the point of emergence directly toward the next available ovule. One pollen tube bypasses three ovules before it grows laterally toward the funiculus of the selected ovule.

(C) Pollen tube emerging on the surface of the septum.

(D) Pollen tube growing on the funiculus and disappearing into the micropyle of the ovule in a wild-type ovary.

(E) Pollen tube growing on the inner side of the ovary wall in a *sin1* ovary.

(F) The growth of pollen tubes is not guided in an ovary of a *bel1* mutant. The pollen tubes grow on all available surfaces including the ovules, which sometimes look almost wrapped by the pollen tubes.

(G) The growth of the pollen tube is no longer directed toward the micropyle in a 47H4 ovary (compare with **[D]**).

(H) 54D12 mutant ovaries usually carry very few intact ovules. In this particular ovary, only one pollen tube is growing. This pollen tube emerges at a position between the fifth and the sixth ovules and then grows back toward the stigma, where it grows up the funiculus of the second ovule.

f, funiculus; mp, micropyle; o, ovule; pt, pollen tube; s, stigma; tr, transmitting tract.

no embryo sacs or a grossly aberrant or intact embryo sac is produced (Figures 4E and 4F; Table 2).

Pollen Tube Path in Ovule Mutants

The pollen tube path was analyzed in all four mutants. The initial growth in the papillar cell wall and inside the transmitting tract is indistinguishable from that in the wild type. However, the first qualitative change in growth behavior, namely, the point of emergence on the surface of the septum, is quite different (Figures 3B and 3C). Whereas in the wild type ~40% of the pollen tubes grow up to the surface at the position of the first ovule, the mutants impaired in ovule development lack this strong preference. In *bel1* and 54D12 mutants, which are somewhat variable with respect to embryo sac development, pollen tubes still show some slight preference for emerging at positions near the stigma. In both the *sin1* and 47H4 mutants, which

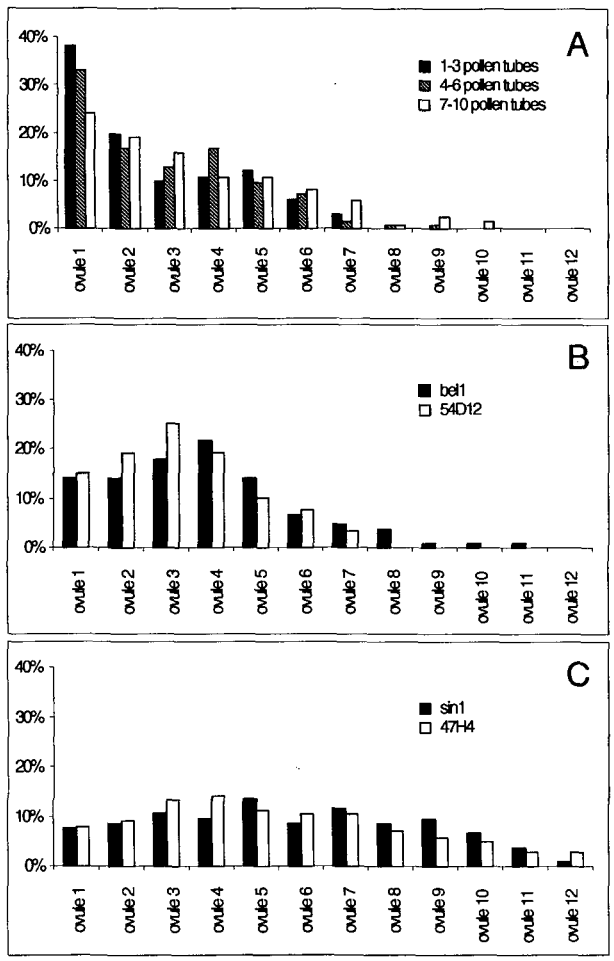

Figure 3. Emergence Pattern of Pollen Tubes in Ovaries of Wild-Type and Ovule Mutant Plants.

The percentage of pollen tubes emerging at various points along the length of the transmitting tract is plotted using the ovules as a scale. Ovules are numbered, with "ovule 1" being closest to the stigma.

do not develop an embryo sac, the pollen tubes emerge with equal probability along the entire length of the septum. These results demonstrate that the directional growth of the pollen tube inside the transmitting tract, as reflected in the pattern of pollen tube emergence, is affected by the presence of intact ovules within the ovary.

In all four ovule mutants, the growth behavior of the pollen tubes is dramatically different from that in the wild type after they emerge on the surface of the septum. Instead of being directed toward a funiculus and the micropyle of an ovule, pollen tubes grow randomly using all available surfaces, including the ovary wall (Figures 2E, 2F, and 2G). Growth on these additional surfaces is not commonly observed in the wild type.

In an effort to identify the tissue inside the ovule that is involved in the guidance of the pollen tube, we took advantage of the variable phenotype of 54D12. In pistils of plants homozygous for the mutation 54D12, ovules that are arrested at different developmental stages compete with each other to attract pollen tubes. Under these competitive conditions, 92% of the ovules with the wild-type phenotype received a pollen tube (Table 2), whereas none of the ovules arrested during megasporogenesis or early embryo sac development were associated with a pollen tube. Of all ovules arrested at intermediate stages of embryo sac development, 28% received a pollen tube. Due to the phenotypic variation among these

(A) The emergence pattern of pollen tubes in the wild type is shown for ovaries in which different numbers of pollen tubes are growing.
(B) The emergence pattern of pollen tubes in ovaries of *bel1* and 54D12 mutant plants. All of the ovaries scored contained between one and three growing pollen tubes. The strong preference to emerge near the stigmatic end of the transmitting tract is reduced in the ovaries of these mutants.
(C) The emergence pattern of pollen tubes in ovaries of *sin1* and 47H4 mutant plants. All of the ovaries scored contained between one and three growing pollen tubes. Pollen tubes growing in the ovaries of these mutants show little or no preference for emerging near the stigmatic end of the transmitting tract.

Table 1. Percentage of Pollen Tubes Guided to a Given Ovule Relative to the Position of Emergence on the Surface of the Transmitting Tract[a]

Number of Pollen Tubes in Ovary	Targeted Ovules							Total No. of Pollen Tubes Scored
	1	2	3	4	5	6	7	
1 to 3 pollen tubes	39	23	17	11	5	3	2	150
4 to 6 pollen tubes	46	22	17	6	6	2	0	143
7 to 10 pollen tubes	44	31	13	8	3	1	0	159

The growth of pollen tubes was analyzed in whole-mount wild-type ovaries that have not been cleared prior to staining (see Figure 2B). This method allows the observation of the path of the pollen tube from the point of its emergence to the targeted ovule.
[a] Pollen tubes were grouped according to the relative proximity of the targeted ovule. Pollen tubes growing toward the next available ovule, relative to their point of emergence, were classified as "targeted ovule 1." Those that bypassed a single ovule were classified as "targeted ovule 2," and so on. This pattern was compared for ovaries bearing a total of one to three, four to six, and seven to 10 pollen tubes. The numbers beneath the ovule markers denote percentage points.

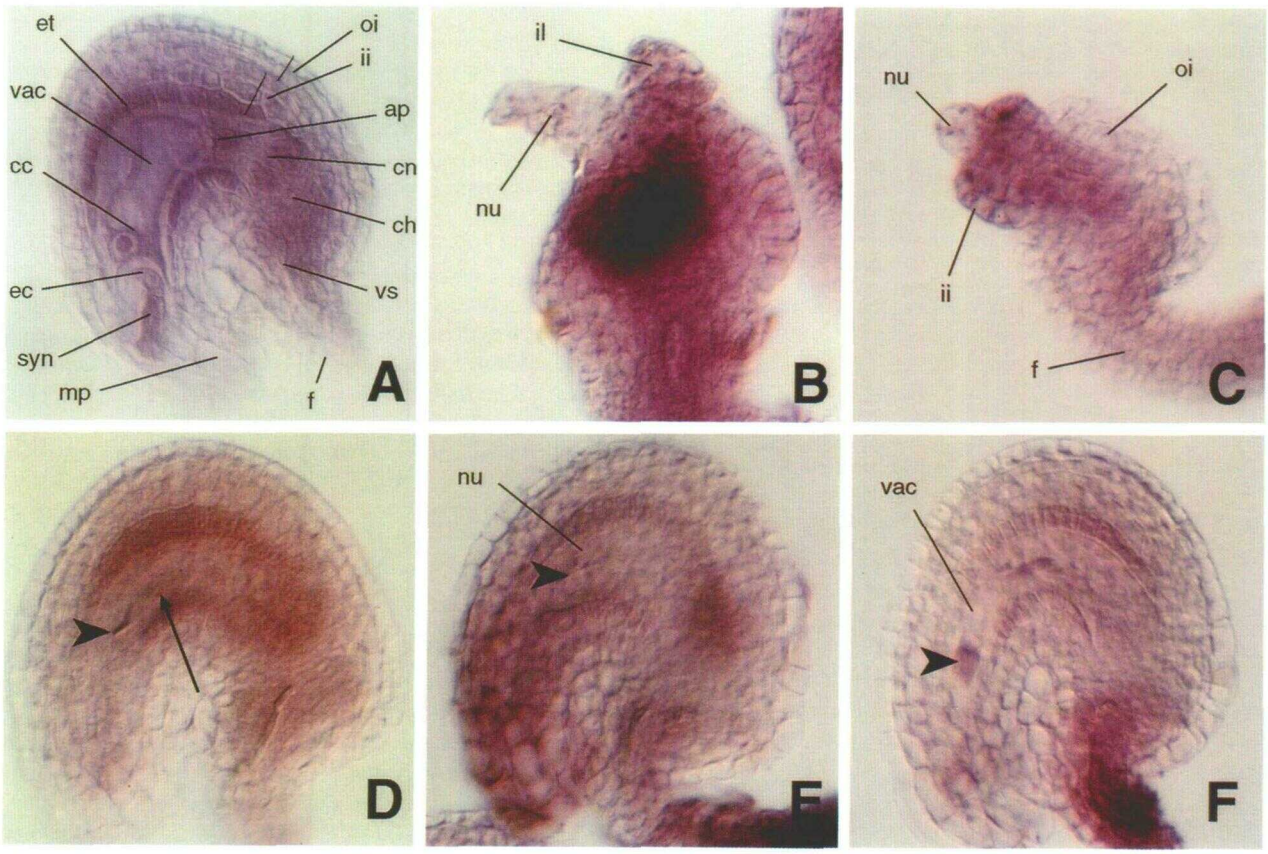

Figure 4. Whole-Mount Preparations of Ovules.

Shown are lateral views of various cleared, whole-mount ovules at a stage corresponding to ~2 days after fertilization. The ovules are oriented with the funiculus to the lower right and the micropyle to the left. The mutants in **(B)** and **(C)** have been described in detail elsewhere (Robinson-Beers et al., 1992; Modrusan et al., 1994). The other mutations were identified in a large-scale, single-line ethyl methanesulfonate mutagenesis experiment designed to identify sterile mutations with a specific defect in ovule development (K. Schneitz, M. Hülskamp, S.D. Kopczak, and R.E. Pruitt, manuscript in preparation). Plants bearing these ovule development mutations showed no other apparent morphological defects.

(A) TH154 mutant. A male-sterile line with normal, fertile ovules is depicted. Development of the megaspore has given rise to the haploid gametophytic tissue (embryo sac). Its constituents, the egg apparatus (two synergids and the egg cell proper), the central cell with its large vacuole, and the antipodal cells, can be detected. The surrounding sporophytic tissue encompasses the outer and inner integuments, including the endothelium, the chalaza with the chalazal nucellus, and the funiculus with the vascular strand.

(B) *bel1.* A nucellus without a mature embryo sac is seen. Instead of the two integuments, only one integument-like structure develops.

(C) *sin1.* No embryo sac can be found. Both integuments are reduced in size.

(D) 47H4 mutant. Inside the nucellus, a degenerated large cell (arrow) with a patch of dense tissue on top (arrowhead) is observed. The sporophytic tissue appears normal.

(E) 54D12 mutant. Shown is an example of a strong phenotype. Instead of an embryo sac, only a patch of dense tissue can be seen in the nucellus (arrowhead). The sporophytic tissue appears normal.

(F) 54D12 mutant. Shown is a representative of an intermediate phenotype. A small, partially developed embryo sac with a vacuole in the center is seen. Instead of the egg apparatus, a patch of darker staining tissue is visible (arrowhead). There is no apparent defect in the sporophyte.

ap, antipodal cells; cc, central cell; ch, chalaza; cn, chalazal nucellus; ec, egg cell; et, endothelium; f, funiculus; ii, inner integument; il, integument-like structure; mp, micropyle; nu, nucellus; oi, outer integument; syn, synergids; vac, vacuole; vs, vascular strand.

defective ovules that received a pollen tube, it is not possible to correlate the guidance competence of the intermediate stages with the development of specific structures of the embryo sac. However, these results show that at some time during embryo sac development, competence to attract pollen tubes is acquired. Furthermore, the highly selective guidance toward only the intact ovules demonstrates that the ovules act functionally as autonomous units with respect to this final aspect of pollen tube guidance. This conclusion is supported by the observation that in pistils that carry only a small number of

Table 2. Correlation of Developmental Arrest of Ovule Development with Guidance Competence in the Mutant 54D12

Ovule Phenotype[a]	No. of Ovules[b]	Associated with Pollen Tube[c]	Not Associated with Pollen Tube[c]
No embryo sac	181 (57%)	0 (0%)	181 (100%)
Partial embryo sac	88 (27%)	25 (28%)	63 (72%)
Wild type	51 (16%)	47 (92%)	4 (8%)

[a] 54D12 mutants show a very variable phenotype with respect to embryo sac development. There was no, partial, or full embryo sac development.

[b] The numbers in parentheses give the relative frequency of the different classes with respect to the total number of ovules scored (320).

[c] The number of ovules in each class that were or were not associated with a pollen tube, respectively. The numbers in parentheses denote the relative frequencies with respect to the total number of ovules per class.

intact ovules, the pollen tube can reverse its basipetal growth on the surface of the septum and grow toward an ovule positioned at the apical end of the ovary (Figure 2H).

DISCUSSION

Pollen tube guidance is a highly regulated process in which four growth phases can be distinguished: (1) growth on the papillar cell, (2) intercellular growth within the transmitting tract, (3) emergence on the surface of the septum, and (4) growth of the pollen tube toward the ovule on the surface of the septum.

The guidance mechanisms in these four phases appear to be different. The initial growth on the papillar cell and the intercellular growth in the transmitting tract are unaffected in all analyzed ovule mutants. The intercellular growth in the transmitting tract is thought to involve predominantly mechanical guidance by the female sporophytic tissue (Capus, 1878; Gueguen, 1901; Heslop-Harrison and Heslop-Harrison, 1986; Heslop-Harrison, 1987). More specifically, according to a model by Sanders and Lord (1989, 1992), the pollen tube is virtually pulled down the transmitting tract by an interaction of the pollen tube with the extracellular matrix of the transmitting tract. Any change from the intercellular growth mode to the surface growth mode seems to require an additional signal (Glenk, 1964; Schwemmle, 1968). In Arabidopsis, this growth change is triggered by a long-range activity that is governed by the ovules. Obviously, the ovules also control guidance of the pollen tubes on the surface of the septum. However, it is not clear whether the nature of the signaling process is the same for both steps. Be that as it may, the long-range guidance activity toward a target, the ovule, rules out a mechanical model and strongly suggests a chemotropic mechanism for the third and fourth guidance steps.

One could envision two types of a chemotropic model with the pollen tubes growing either along a concentration gradient toward the source of the signal or along a single, invariant, and target-induced track of signal molecules (Heslop-Harrison, 1987). Two observations support a concentration gradient hypothesis. (1) An individual ovule can attract a pollen tube from a variety of different emergence points. In the wild type, the position of a given emergence point is usually, although not always, close to the selected ovule. In a given mutant case, however, pollen tubes that have emerged may grow from any position toward intact ovules. (2) The precise path followed by a pollen tube on the funiculus/ovule varies greatly from one ovule to another. This is not consistent with the presence of a well-defined track of recognition molecules at an invariant location on the surface of the tissue.

It has been suggested for more than 100 years that the most likely explanation for pollen tube guidance is a chemotropic mechanism (Mascarenhas and Machlis, 1962; Rosen, 1975; Mascarenhas, 1978). Although in vitro studies have found a chemotropic response of pollen tubes to different parts of the pistil as well as to a number of chemical agents, no conclusive evidence could be obtained to support a chemotropic model (Heslop-Harrison and Heslop-Harrison, 1986). Part of the debate over pollen tube chemotropism arises from the intrinsic difficulty in deciding whether pollen tube guidance is a consequence solely of nutritional growth support or is orchestrated by a specific signal (Mascarenhas, 1978; Heslop-Harrison, 1987). However, the extensive random growth of pollen tubes inside the ovary in ovule mutants indicates that the guidance of the pollen tube on the surface of the septum is independent of nutritional support in Arabidopsis.

Sporophytic mutations that specifically affect embryo sac development without an apparent defect in the sporophytic tissue of the ovule show a strong guidance phenotype. Hence, based on our morphological criteria, it is possible that the sporophytic tissue of the ovule plays no or no essential role in guidance. By the same token, a proper female gametophyte/embryo sac appears to be crucial for the signaling process to function. This suggests that at least some of the genes specifically involved in guidance may be gametophytically active genes.

METHODS

Growth of Plants

All experiments were performed using the Landsberg ecotype of *Arabidopsis thaliana* bearing the *erecta* mutation. Plants were grown in a 1:3 mixture of perlite and Metro Mix 350 (Grace-Sierra, Milpitas, CA) and fertilized weekly with one-quarter strength Hoagland's solution. All plants were grown in Percival I-37LLVL (Percival Scientific, Boone, IA) growth chambers with constant illumination at 25°C and 70% relative humidity.

Microscopy and Graphic Work

For light and fluorescence microscopy, a Microphot-FXA microscope (Nikon Inc., Melville, NY) with differential interference contrast optics and an epifluorescence attachment was used. Photographs were taken on Kodak Ektachrome color slide films (Eastman Kodak, Rochester, NY). Photographs were processed for publication using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) and Aldus Freehand (Aldus Corp., Seattle, WA) software on a Macintosh IIfx computer (Apple Computer Inc., Cupertino, CA). Prints were generated on a Tektronix Phaser IISDX color printer (Tektronix Inc., Wilsonville, OR).

Scanning Electron Microscopy

Pistils were removed from the flowers and partially dissected. The primary fixation was done in 3% glutaraldehyde in cacodylate buffer (50 mM sodium cacodylate, pH 7.0). After an initial fixation for 2 hr at room temperature under a gentle vacuum, pistils were kept at 4°C overnight. Specimens were then washed three times in cacodylate buffer and post-fixed in 2% osmium tetroxide in cacodylate buffer overnight at 4°C. The fixed pistils were rinsed three times in cacodylate buffer, dehydrated in a graded ethanol series, and critical point dried in liquid carbon dioxide. The dry pistils were mounted on stubs and further dissected. After sputter coating with gold, pistils were analyzed with a scanning electron microscope.

Whole-Mount Ovule Preparations

Fixed ovules were stained in Mayer's hemalum (Sass, 1951) and processed essentially as described by Stelly et al. (1984) with the exception that methyl benzoate was used as the clearing agent (Webb and Gunning, 1990).

Pollen Tube Staining in Ovaries

The path of pollen tubes inside the pistil was visualized by staining whole pistils with aniline blue (Martin, 1959). First, the ovary wall of the pistil was removed, and the ovules were folded to the side. Then the pistils were cleared in 10% chloral hydrate at 65°C for 5 min, washed with tap water, treated with 5 M NaOH at 65°C for 5 min, washed with tap water, and stained in 0.1% aniline blue in phosphate buffer, pH 8.3. Alternatively, the pistils were immediately stained with aniline blue after dissection.

The emergence pattern of pollen tubes was determined by emasculating and hand-pollinating flowers with very few wild-type pollen grains. In whole-mount preparations of noncleared, aniline blue–stained pistils, the number of pollen tubes that emerge at the position of a given ovule was counted. To standardize the method, ovaries containing one to three, four to six, and seven to 10 pollen tubes were grouped and plotted separately (see Figure 3A). Each graph is based on a minimum of 100 emergence points. For a comparison of wild-type ovaries and ovule mutants, only ovaries that contained between one and three pollen tubes were used.

To determine both the ovule phenotype and its association with a pollen tube, pistils were dissected and stained with aniline blue as previously described. In individual pistils, all ovules that were associated with a pollen tube were recorded. Subsequently, the pistils were cleared with methyl benzoate, and the ovule phenotype was determined.

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